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A Technical Review of *Haematococcus* algae: Metabolic and Health Effects of Astaxanthin

Summary: A growing body of scientific literature is demonstrating that the carotenoid, astaxanthin, surpasses beta-carotene, zeaxanthin, canthaxanthin, vitamin C and vitamin E in antioxidant potential. Animal studies have also shown that astaxanthin can protect skin from the damaging effects of ultraviolet radiation, protect against chemically induced cancers, increase high density lipoproteins and enhance the immune system. Human-grade *Haematococcus* microalgae is produced in Kailua-Kona, Hawaii.

Carotenoids

Carotenoids are a family of over 700 natural lipid-soluble pigments that are primarily produced only by phytoplankton, algae, and plants. The carotenoids are responsible for the wide variety of colors they provide in nature, most notable are the brilliant yellow, orange and red colors of fruits, leaves, birds, and aquatic animals. In their native function in plants and alga, carotenoids participate as secondary light-harvesting and antioxidant molecules in the photosynthetic process. Some animals are capable of converting carotenoids to other forms, but animals cannot synthesize them de novo. Plants, algae, and some fungal and bacterial species produce carotenoids, whereas animals must obtain them from their diet. The general distribution and metabolic pathways of carotenoids has been extensively detailed in other reviews (Goodwin 1984, Davis 1985, Matsuno and Hirao, 1989).

Interestingly, animals have adapted to exploit the potent antioxidant properties of carotenoids. One familiar example is seen in the cold water fish that accumulate the carotenoid, astaxanthin, from their diet. The astaxanthin is then deposited in the muscle actomyosin to protect lipid tissues from peroxidation. We know this carotenoid as the beautiful pinkish-red hue in the flesh of salmon and trout (Tacon, A., 1981, Craik, J. 1985; Torrissen, O.J., 1984). Farm-raised salmonids have astaxanthin supplied in their artificial pellet diet, otherwise their flesh would be white and have a much lower market value. However, it has been recently demonstrated that astaxanthin has functions beyond flesh pigmentation. In fact, it has been well documented that astaxanthin is actually an essential vitamin for aquaculture-raised fish. An extensive body of data emphasizes the vital role of carotenoids in the physiology of fish and concludes that carotenoids are essential nutrients that should be included in all artificial aquaculture diets at a minimum level of 10 ppm (Craik, 1985; Torrissen, 1990; Grung et al. 1993). Interestingly, a recent groundbreaking study in Norway by Christiansen and his colleagues demonstrated that Atlantic salmon fry have a crucial growth and survival requirement for astaxanthin in their diet. Thus, Atlantic salmon have the distinction as being the first salmonid species for which astaxanthin has been shown to be an essential vitamin, with absolute minimum levels being about 5.1 ppm. Other studies have linked a role for astaxanthin in the regulation or metabolism of vitamin E (α-tocopherol), vitamin A and retinol (Christiansen 1994, 1995a, 1995b).

Natural Sources of Astaxanthin

Astaxanthin is ubiquitous in the nature, especially in the marine environment. It is probably best known for eliciting the pinkish-red hue to the flesh of salmon and trout, as well as shrimp, lobsters and crayfish. Since these animals are unable to synthesize astaxanthin *de novo*, their carotenoid pigments must be supplied in their diet. In the marine environment, astaxanthin is biosynthesized in the food chain within microalgae or phytoplankton as the primary production level. These microalgae are consumed by zooplankton, insects or crustaceans which accumulate astaxanthin and in turn are ingested by salmonids (Kitahara 1984 and Foss et al., 1987). Land animals and birds obtain their carotenoids directly from algae, plants, or insects that have fed on these sources.

Natural sources of astaxanthin are numerous but nearly all are found in very low concentrations. Astaxanthin has been extracted from processed crustacean wastes of krill, shrimp, crab and crawfish. These extracts have been used in the diets of farm-raised salmon and trout, since salmonids cannot themselves produce the red carotenoid found in the muscle. However, crustacean waste products (oils and meals) generally contain less than 1000 ppm of astaxanthin. Furthermore, crustacean sources contain high amounts of moisture, ash, fluoride and chitin that limits the percentage of these products that can be included in salmonid feeds. Another natural source of astaxanthin has been derived from *Phaffia rhodozyma*, however the concentrations are typically 0.5% (5,000 ppm). The flowers of the plant, *Adonis annua*, are unusual in that they contain relatively high amounts of astaxanthin. However, the plant is not grown as a commercial source of astaxanthin due to the low yields.

By far, the algae *Haematococcus pluvialis* provides the most concentrated natural source of astaxanthin. *Haematococcus pluvialis* also referred to as *Haematococcus lacustris* or *Sphaerella lacustris*, is a ubiquitous green alga of the order Volvocales, family Haematococcaceae as described in the following table..

Classification

Phylum:	Chlorophyta
Class:	Chlorophyceae
Order:	Volvocales
Family:	Haematococcaceae
Genus:	Haematococcus
Species:	pluvialis

The alga occurs in nature worldwide, where environmental conditions for its growth are favorable. *Haematococcus* algae has a number of different forms during its life cycle, and is normally found in ephemeral pools of fresh water where temperatures are cooler. Under these conditions, *Haematococcus* is a motile alga about 20-30 um in size, which utilizes the available nitrate, phosphate, and other nutrients to grow and reproduce. However, when nutrients become limiting or the pool begins to dry the alga form a protective cell wall and encyst. Massive amounts of astaxanthin are produced, and the cells undergo a dormant stage until the next influx

of water and nutrients. Cells can remain viable in this encysted stage for decades. Red cysts are significantly more resistant to photoinhibition and oxygen radicals than green cells, strongly indicating a protective roles for astaxanthin (Kobayashi et al., 1992a).

Natural Astaxanthin from Microalgae: A Breakthrough Technology

Advanced technology was developed to grow *Haematococcus* algae using pure culture conditions. The algae is cultivated employing a proprietary closed culture technology known as PhytoMax PCS (Pure Culture System) which automatically regulates cell culture conditions before transfer to open ponds for the final stage of astaxanthin production. The system harnesses the unique properties of the algae to produce the highest concentration of natural astaxanthin ever achieved. Lots are standardized and formulated to contain 1% (10,000 ppm) astaxanthin in a predominately esterified form. The carotenoid fraction of the microalgae contains about 70% monoesters of astaxanthin, 10% diesters of astaxanthin, 5% free astaxanthin, and the remaining 15% consists of a mixture of β-carotene, canthaxanthin, lutein and other carotenoids which are also beneficial as antioxidants and provitamin A activity. These esterified forms of astaxanthin provide higher stability and are similar to the composition of krill and crawfish, but at a 150-fold higher concentration (Lambertsen et al., 1971, Foss et al., 1987, Maoka, T. et al., 1985, Yamaguichi et al., 1983). Thus, the microalgae could be described as a "concentrated" krill or crawfish extract.

All media ingredients for the cultivation of the algae are food grade or higher quality. The algae is pasteurized to prevent any possible microbial contaminants. Only reliable manufacturers that include specifications for heavy metals and other possible contaminants are utilized for the culture nutrients. Solvents, pesticides, herbicides or toxic substances are not used during cultivation or manufacturing of the product. Animal studies have proven that *Haematococcus* algae is safe, it has never been associated with any toxicity in the reported literature or in field studies. *Haematococcus* algae has undergone the scrutiny of Japanese regulatory agencies and has been approved as a natural pigment for foods as well as in fish feeds.

Haematococcus algae is now commercially available as a human supplementation form and provides the richest natural source of astaxanthin in the world. A different formulation of *Haematococcus* algae has already gained wide acceptance in the aquaculture markets as a pigmentation and vitamin source for salmon, trout, shrimp and ornamental fish. The production process includes a technique which "cracks" greater than 95% of the cells to enable maximum bioavailability, resulting in a fine dark red powder.

Safety Studies of *Haematococcus* Algae Meal

Acute oral toxicity studies have been conducted on Charles River CD rats. The dosage level was 5,000 mg *Haematococcus* algae/kg and was administered as a 0.5% aqueous methylcellulose solution. Each lot was administered to separate groups of 10 rats that consisted of five males and five females. Groups for each treatment effect were evaluated for mortality, pharmacotoxic signs, body weights, and necropsy examinations during the 13-day study. The results demonstrated that the LD_{50} value of each lot was greater than the administered dose of

5,000 mg/kg. No visible abnormalities were observed, nor differences in body weights during the study. The postmortem examination did not reveal any abnormalities in rats sacrificed at the end of the study.

Additional acute oral toxicity studies were conducted with both male and female mice. *Haematococcus* algae meal was suspended in distilled water for injection to give a 30% solution (w/v). The solution was forced by oral administration once using a gastric probe. The dosages ranged from 10,417-18,000 mg/kg, no mortalities were observed. The postmortem examination did not reveal any abnormalities in the rats that were sacrificed at the end of the study. The oral LD_{50} was judged to be 18,000 mg/kg or above.

A mutagenicity test using *Salmonella typhimurium* strain TA100, TA1535, TA98, TA1537, TA1538 and *E. coli* WP2 uvr A. A sample of *Haematococcus* algae meal was formulated into a 50 mg/ml solution of dimethyl sulfoxide. The formulation was spread onto the test petri plates in the presence of the microbial cultures with positive controls. The positive controls 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, 1-ethyl-2-nitro-3-nitrosoguanidine, 9-aminoacridine, 2-aminoanthracene, and 2-nitrofluorene showed a remarkable increase in the number of reverent colonies compared with the solvent control. The *Haematococcus* algae meal sample showed no significant increase in the number of reverent colonies in every case compared to the solvent control. This demonstrated that the mutagenicity of the *Haematococcus* algae meal sample under the employed conditions were negative.

Fish tissues from a *Haematococcus* algae feeding study of rainbow trout were analyzed for toxic effects and neoplasia. All tissues examined were normal in appearance with no indication of disease, toxicity or neoplasia. All fish examined were in excellent nutritional status with abundant body fat. Gross findings indicate that no adverse effects on health were observed from *Haematococcus* algae meal as the dietary source of astaxanthin.

There are no carcinogens or compounds that may degraded or metabolized to carcinogens used in the manufacturing process or known within *Haematococcus* algae. The following Table lists the maximum tolerances of the product as designated by the Federal Food and Drug Administration.

Heavy metal tolerances

Heavy Metals (as lead),	<10.0 mg/kg
Mercury	<1.0 mg/kg
Cadmium	<0.5 mg/kg
Arsenic	<2.0 mg/kg
Lead,	<5.0 mg/kg

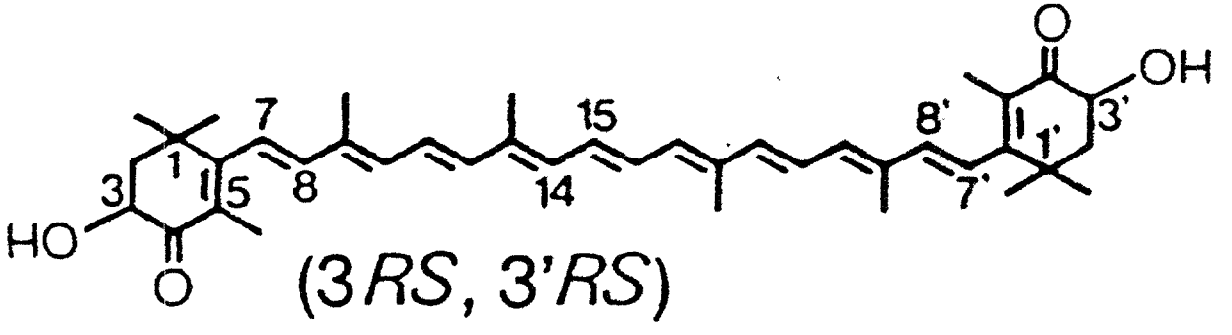
Chemistry of Astaxanthin

In their native niche of plants and alga, carotenoids participate as secondary light-harvesting pigments in the photosynthetic process of plants. Carotenoids are synthesized through the isoprenoid biosynthetic pathway, which is also responsible for such diverse compounds as

prostaglandins, steroids, sterols, vitamins A, D, E, and K. The pathway initiates at acetyl-Co-A and proceeds through phytoene, lycopene, β-carotene, and canthaxanthin before the last oxidative steps to astaxanthin. The biosynthesis of astaxanthin is one of the most expensive molecules, in metabolic terms, which an algae or plant cell ever produces. Thus, it must play very critical roles for cells otherwise there would be a strong selection against its synthesis.

The astaxanthin molecule has two asymmetric carbons located at the 3 and 3" positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the exact way that the hydroxyl groups (-OH) are attached to the carbon atoms at these chiral centers. If the hydroxyl group is attached so that it projects above the plane of the molecule it is said to be in the R configuration and when the hydroxyl group is attached to project below the plane of the molecule it is said to be in the S configuration. Thus, the three possible enantiomers are designated R,R', S,S' and R,S' (meso). Free astaxanthin and its mono- and diesters from *Haematococcus* algae have optically pure (3S,3'S)-chirality (Grung et al., 1992; Renstrom et al., 1981). The long system of double bonds between the ring structures is the conjugated polyene system, and plays important roles in the antioxidant features of the molecule.

Astaxanthin



Carotenoids with these hydroxy-, keto-, methoxy-, epoxy-, or carboxyl- groups are collectively called 'xanthophylls". Whereas unsubstituted carotenoids are called "carotenes", exemplified by beta-carotene. Fatty acids are esterified to the 3 or 3' hydroxyl groups, resulting in mono and diesters of astaxanthin. Esterified astaxanthin is more soluble in the cellular environment and inherently more stable to oxidation, thus it makes sense that the esterified form is the predominant type found in nature.

Complexes of carotenoids and proteins generally called "carotenoproteins" are widely found in crustaceans. Astaxanthin is a red carotenoid pigment, but when complexed with various proteins, the light absorbance shifts and causes crustaceans to range in color from green, yellow, and blue to brownish. The red color of cooked crustaceans is actually produced when these proteins denature from the heat and release astaxanthin from their protein group (Muriana et al., 1993 and Nur-E-Bordan et al., 1995, Britton et al., 1981).

Antioxidant Properties of Astaxanthin

Although oxygen is required for metabolic functions, it presents severe challenges to cells. Animals have developed sophisticated systems of arteries, veins and capillaries to deliver and regulate oxygen-rich blood to every cell of the body. However, an equally elaborate cascade of metabolic enzymes (superoxide dismutase, catalase, glutathione peroxidase) and antioxidants (carotenoids, glutathione, ascorbic acid, tocopherols) is necessary to constantly to rid our cells of dangerous species of oxygen-derived molecules. Harmful varieties of oxygen called "singlet oxygen" and "free radicals" are unstable molecules that contain an unpaired electron. These species are formed as a consequence of photooxidation, physiological stress and normal immune system functions. For example, following phagocytosis of microbes by the immune system, a respiratory burst by the phagocytes produces high levels of free radicals to aid in their killing and degradation. These excess free radicals must be neutralized such that host cells are not damaged. Otherwise, free radical damage can lead to protein scission, disulfide crosslinking, lipid-protein crosslinking, nucleic acid damage, amino acid oxidation, lipid-lipid crosslinking, as well as fatty acid peroxidation.

We normally have a balance of free radicals and an arsenal of various antioxidants to counter them, but poor nutrition or disease can upset this equilibrium. Many theories suppose that an upset oxidative balance can be a contributing factor in such conditions as rheumatoid arthritis, heart disease, Parkinson's disease, Alzheimer's disease, cancer and stroke. Free radicals have an inordinate affinity to attack unsaturated fatty acids, the principle component of cell membranes, thereby creating more fatty acid radicals in a chain reaction. Carotenoids are distinguished by their capacity to interact with chemically reactive species of oxygen such as singlet oxygen and free radicals. Due to its particular molecular structure, astaxanthin has a very potent neutralizing or 'quenching" effect against singlet oxygen as well as a powerful scavenging ability for free radicals. Thus, astaxanthin serves as an extremely effective antioxidant protector against these reactive species (Kurashige et al. 1990; Jorgensen, 1993; Miki, 1991, Di Mascio, 1989, Terao, 1989).

In fatty acid peroxidation, free radicals attack unsaturated fatty acids of cell membranes and create more fatty acid radicals. These, in turn attack additional fatty acids in a chain reaction which cause general cellular damage. A particular variety of oxygen called "singlet oxygen" can also lead to formation of free radicals, causing cellular injury. Due its particular polyene molecular structure and oxo groups, astaxanthin has a strong neutralizing or "quenching" effect against singlet oxygen and a powerful scavenging effect against free radicals, thus serving as a very effective antioxidant protector for these reactive species (Kurashige et al. 1990; Jorgensen, 1993; Miki, 1991). Additionally, xanthophylls such as astaxanthin possess the ability to act as chain-breaking antioxidants in the peroxidation of membranous phospholipids (Lim, 1992).

Conventional chain-breaking antioxidants such as tocopherols (vitamin E) trap peroxyl radicals by donating a hydrogen atom. However, carotenoids exert their antioxidant effect by trapping the peroxyl radical (**ROO•**) within the conjugated polyene system. The resulting radical is resonance-stabilized in this polyene system which leads to termination of the chain reaction (Terao J., 1989, Jorgensen, 1993). The unique structure of astaxanthin scavenges lipid radicals and effectively breaks the peroxide chain reaction (Terao, 1989).

ROO• + carotenoid → ROO-Carotenoid•

The resulting carbon-centered radical is stabilized by resonance of the conjugated polyene system. Addition of a second peroxyl radical to the carbon-centered radical yields non-radical polar products, resulting in an overall trapping of two peroxyl radicals per carotenoid molecule consumed:

ROO-Car• + ROO• → inactive polar products

The remarkable effectiveness for inactivating active oxygen species ($^1O_2$) and in the process forming triplet carotenoids very rapidly is shown in the following reaction:

$^1O_2$ + carotenoid → $^3O_2$ + ^{3}carotenoid

Triplet carotenoids can simply revert to the ground state with the liberation of a small amount of heat. It is generally accepted that carotenoids with an increasing number of conjugated bonds in the polyene system are associated with a greater quenching activity against $^1O_2$ (Foote and Denny, 1968; Krinsky, 1989; Truscott, 1990).

Antioxidant Studies

Researchers have developed various methods to measure the antioxidant capacity of carotenoids. Some of these assays are conducted in test tubes (in vitro) to better control conditions, as opposed to within cell cultures directly (in vivo). Typically, a chemical that generates radical cations, singlet oxygen or peroxides is mixed with a substrate such as a fatty acid that can become readily oxidized. When the reaction rate is determined, carotenoids can then be added to determine how it quenches, or slows the peroxidation rate of the fatty acid.

Numerous studies exist demonstrating the potent radical scavenging and singlet oxygen quenching properties of astaxanthin (Haila Katri, 1997;Woodall, 1997;Nakagawa, 1997;Oshima, 1993; Tinkler, 1994). In one of the first antioxidant studies with astaxanthin, Di Mascio utilized a chemoluminescence technique to demonstrate that its superior singlet oxygen quenching ability compared to other carotenoids. He concluded that the effectiveness and potency of astaxanthin was related to its ability to function at the oxygen concentrations present in tissues to thereby help prevent cellular damage (Di Mascio, 1989). In another study, it was demonstrated that astaxanthin was more effective in neutralizing free radicals than beta-carotene (Terao, 1989).

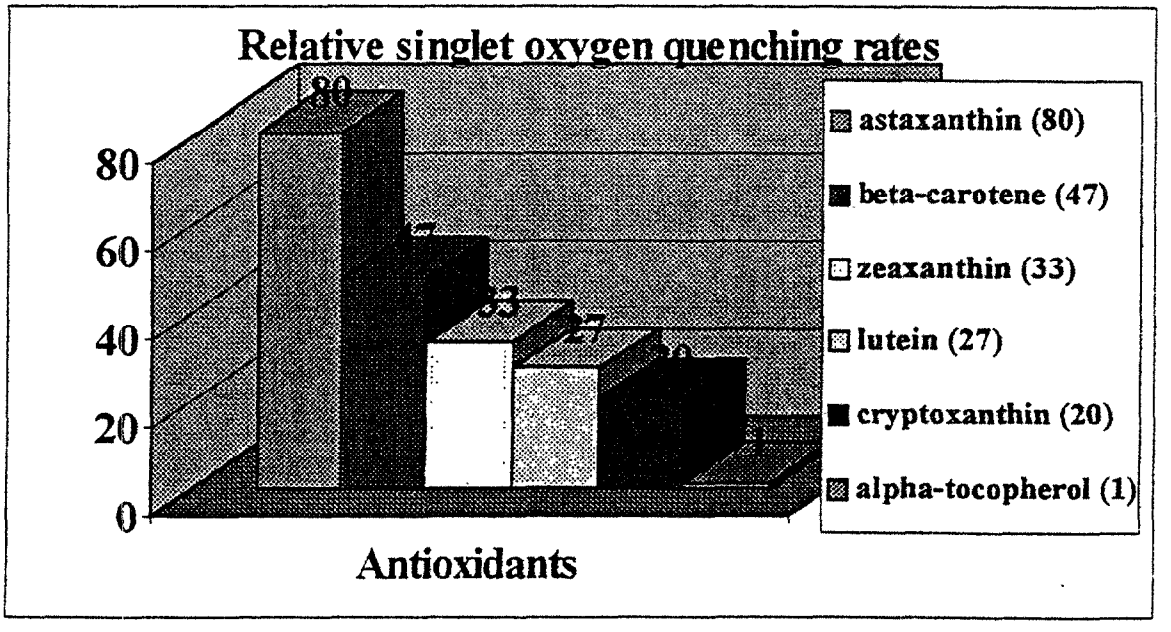
It has been shown that astaxanthin retards the peroxidation of unsaturated fatty acids more efficiently than canthaxanthin, beta-carotene or zeaxanthin (Jorgensen, 1993). The scavenging of lipid radicals by astaxanthin effectively results in disruption of the oxidative chain reaction. Additionally, xanthophyll carotenoids containing keto groups at the 4 and 4' positions in astaxanthin serve as more potent free radical scavengers than other carotenoids in the

prevention of lipid peroxidations (Terao, 1989). Although various researchers use different assay systems, astaxanthin was approximately 10 times stronger in terms of antioxidant activity than other carotenoids such as zeaxanthin, lutein, tunaxanthin, beta-carotene and canthaxanthin (Miki, 1991) and from 80-550 times greater than alpha-tocopherol (Di Mascio, 1989; Ranby and Rabek 1978; Shimidzu, 1996). Thus, astaxanthin has been proposed as the "super vitamin E" (Miki, 1991).

Singlet oxygen quenching constants (Kq 10^{-9} $M^{-1}S^{-1}$)

Carotenoid	Quenching Rate Constant (kq)
Astaxanthin	24
Beta-carotene	14
Zeaxanthin	10
Lutein	8
Cryptoxanthin	6
Alpha-tocopherol	0.3

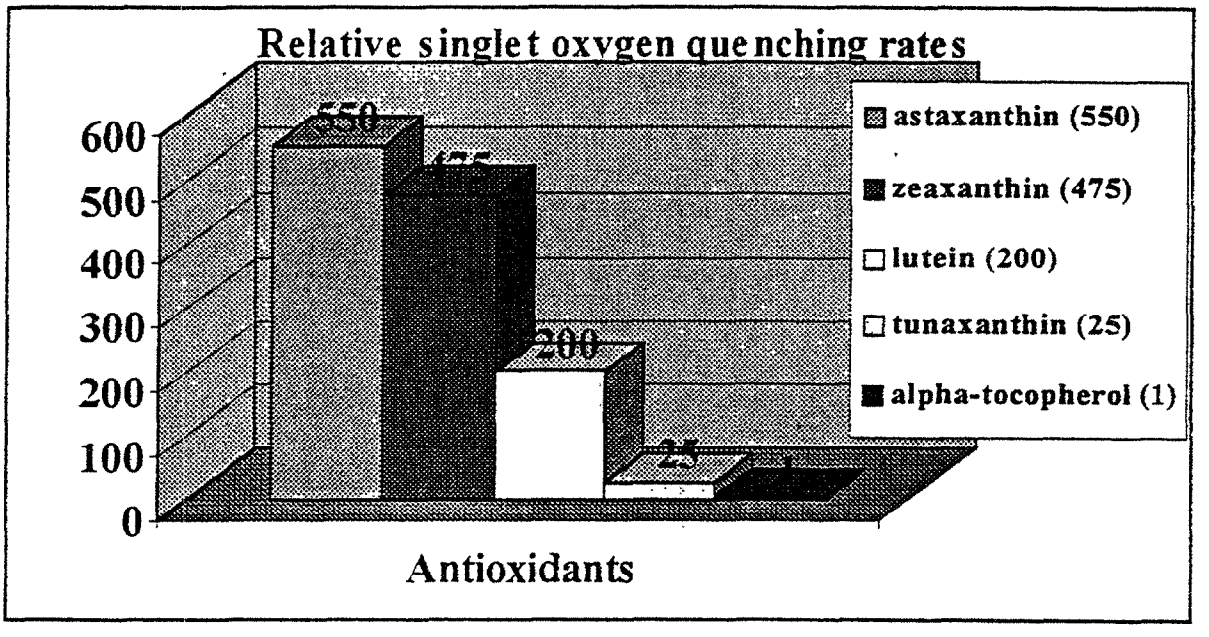
(Adapted from Di Mascio et al 1989)



Singlet oxygen quenching activities (Kq $10^{-9}M^{-1}S^{-1}$)

Carotenoid	Quenching Rate Constant (kq)	Relative to tocopherol
Astaxanthin	2.2	550
Zeaxanthin	1.9	475
Lutein	0.80	200
Tunaxanthin	0.15	25
Alpha-tocopherol	0.004	1

(adapted from Shimidzu, 1996)



In cell culture studies, similar results demonstrate the efficacy of astaxanthin as an antioxidant and chain-breaking molecule in the peroxidation of membranous phospholipids (Lim, 1992). In one study, primary cultures of chicken embryo fibroblasts (CEF) were oxidatively stressed by exposure to the radical generator, paraquat, and various levels of astaxanthin from 0.1-10 nM were added to ascertain the antioxidant effect. Activities of the antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase were measured as indices of oxidative stress. Without astaxanthin, paraquat increased the activities of SOD and catalase more than two-fold, and decreased the activity of glutathione peroxidase by more than 50% indicating high oxidative stress. Protection against paraquat-induced oxidative stress was observed at all levels of astaxanthin tested, demonstrating its effectiveness as an antioxidant in this model (Lawlor and O'Brien, N.M., 1994). Astaxanthin furnishes more protection to rat liver

microsomes undergoing radical-initiated lipid peroxidation than beta-carotene or vitamin E (Palozza, 1992; Nishigaki, 1994).

Many antioxidant studies are conducted under conditions of low vitamin E (tocopherol) or vitamin A to assess the actual effects of the added carotenoids. In vitamin E-deficient rats, astaxanthin protects the mitochondria from damage caused by lipid peroxidation, and the antioxidant activity is greater than vitamin E (Kurashige, 1990). Ultraviolet radiation has long been known to cause epidermis photoaging and skin cancer. Special hairless mice sensitive to UV light are used to understand the effects of antioxidants and light-induced oxidation. Using this model, significantly less damaging metabolites accumulate in the presence of astaxanthin compared to retinol or beta-carotene suggesting a specific effect on enzymes called "transglutaminases" (Savoure, 1995). In rat kidney fibroblasts, astaxanthin exhibited superior protection against UVA light-induced oxidative stress compared to lutein and beta-carotene (O'Connor, 1998).

Vitamin A, vitamin C, vitamin E (α-tocopherol) and carotenoids all function as antioxidants, both in vivo and in vitro. It is worthy to note that the antioxidants work in synergism, an absence of any one of them (or other participating components such as zinc, selenium, copper) reduces the efficiency of the entire cascade significantly (Challem, 1998).

Metabolic Effects of Astaxanthin

In humans and other animals, carotenoids are essential for proper health of the eye. The macula is the small central part of the retina, an area of about 2 millimeters in diameter directly behind the lens of the eye that produce sharp vision need to read and see fine details clearly. It functions as a screen onto which all visual images are projected and also determines our ability to see colors. The blue, indigo and purple end of the visible light spectrum is composed of photons that have the highest levels of energy. Unfortunately, the constant processing of blue light generates high levels of singlet oxygen and free radicals that can cause damage of the lipid components through photooxidation. Additionally, the macula is especially rich in oxygen and contains the highest concentration of polyunsaturated fatty acids of any tissue in the human body, thus it is especially susceptible to oxidation. However, carotenoids within the macula absorb the blue light spectrum, quench free radicals and thereby prevent damage and peroxidation of the photoreceptor membranes.

Clinical studies show that light injury is a cause of age-related macular degeneration (AMD), an irreversible blindness, because of the cumulative insult leading to a gradual loss of photoreceptor cells. In parallel, older people have a lower level of carotenoids in their eyes. AMD is the leading cause of irreversible blindness among older Americans and causes partial loss of vision in 5% of the general population. Those that have progressed to AMD have some peripheral vision but cannot see straight ahead with precision, eventually it leads to blindness. Epidemiology studies have indicated that the consumption of high levels of carotenoids leads to a reduced risk of AMD. Those people in the highest quintile of carotenoid intake had a 43% lower risk for AMD than those in the lowest quintile. AMD is not reversible, but carotenoids in the diet may decrease the risk of AMD or slow the progression of the disease. Additionally, antioxidant

carotenoids may maintain the integrity of the choroidal blood vessels that supply the macular region of the retina.

Unlike beta-carotene, astaxanthin is able to readily cross the blood-retinal brain barrier and protect the retina against photo oxidation and loss of photoreceptor cells. Astaxanthin has not been shown to crystallize in the retina, though it has been problematic with canthaxanthin in the past. Furthermore, astaxanthin has the ability to protect the neurons of the retina as well as the central nervous system, especially the brain and spinal cord, from damage caused by free radicals (Seddon et al. 1994; US Patent 5,527,533). In animal tests, seven albino Lewis rats were first fed a normal diet and placed on a twelve hour cycle of light and darkness for 14 days. Four rats were then administered intraperitoneal injections of astaxanthin corresponding to 37.5 mg astaxanthin/kg of body weight at 12 hour intervals. All seven rats were then exposed to 180-200 ft-candle (1800-2000 lux) green-filtered fluorescent light at 490-580 nm for 24 hours. The rats were then kept in the dark for a two-day recovery period and euthanized for analysis of the retinas. By measuring the thickness of the outer nuclear layer (ONL) of the retina, a quantitative determination of the photoreceptor cell degeneration could be made. It was found that control rats without treatment or photic injury had an ONL measurement of 45 microns, whereas the group receiving photic injury without astaxanthin supplementation had an ONL measure of 32 microns. The ONL measurement of rats receiving astaxanthin and photic injury had an ONL measurement of 42 microns, which showed that administration of the carotenoid provides a significant protection to receptor cells from photic injury. The astaxanthin protected the photoreceptors in each eye of the four quadrants and in the whole eye as well. A similar follow-up study was conducted with oral dosing of astaxanthin to measure the effects of photic injury on rhodopsin levels in the eye. It was found that rhodopsin levels in the retinas of control rats fell for six days following photic injury, then began to recover. After 6 hours of photic injury, the rhodopsin level of control rats was 0.75 nmol, and continued to decrease to 0.5 nmol after 6 days. The level improved to 0.8-0.85 nmol after 13 days from the initial photic insult. In contrast, the astaxanthin-treated rats had a rhodopsin level of about 1.15-1.2 nmol at the 6-hour post injury stage. Additionally, the rhodopsin did not decrease over the subsequent 6 days, but increased to a level of about 1.25 nmol and remained essentially constant through day 13 after photic injury. The authors state that the astaxanthin not only protects the receptor cells from photic injury but also ameliorates the effects of the damage since the rhodopsin levels never decrease, but rather increase over the recovery period (US Patent 5,527,533).

Carotenoids also have a physiological effect in cell growth and metabolism. High-density lipoprotein (HDL) is a complex of lipids and proteins that functions as a transporter of cholesterol in the blood. High levels of HDL are associated with a decreased risk of atherosclerosis and coronary heart disease, whereas high concentrations of LDL have an opposite correlation. Male Wistar rats fed 1000 ppm dietary astaxanthin had increased levels of HDL in their plasma, whereas beta-carotene did not have the effect (Murillo, 1992). Additional studies are in progress, but it is it is speculated that carotenoids my decrease the oxidation of these lipid-carriers and thereby reduce the risk of atherosclerosis.

Gap junctions are relatively non-specific pores between cells that are "gated" such that they can open or close in response to certain stimuli. The plasma membranes of adjacent gap cells

contain hexagonal structures made from the protein, connexin. When these gap cells are aligned, a connection is made through which small molecules or ions can flow between cells. These functions are especially in the propagation of nerve impulses. Carotenoids are known to protect cells against chemically induced carcinogenic transformations through the enhancement of gap junctional communication between cells. Chemoprevention activity strongly correlates to the expression of the gene, connexin43, coding for a gap junctional protein (Bertram J.S. et al., 1991). Gap junctional communication (GJC) has been linked to increased growth control and evidence indicates that chemopreventive carotenoids increase expression of this gene and act as potential chemoprotective agents (Zhang L.X. et. al.1991; King T.J. et.al. 1997). This has led to the theory that carotenoids enhance or expand gap junction communication which in turn serves as a conduit for growth regulatory signals (Bertram, 1991; Zhang et al, 1992). The effect on gap junctions is also partially explained by the finding that astaxanthin functions as a membrane stabilizer, essentially acting as trans-membrane rivets between lipid bilayers (Woodall, 1997; Milon, 1986).

Astaxanthin in Cancer Deterrence

Epidemiological studies consistently demonstrate a correlation between carotenoid intake and the reduced incidence of cancer, and increased resistance to viral, bacterial, fungal and parasitic infections. Studies indicate that the mechanism for this protective attribute is partly due to the direct enhancement of the immune response by carotenoids. Additionally, anticarcinogenic effects of carotenoids may be partly attributable to its antioxidant effect, insofar as oxygen radicals are related to the process of cancer initiation and propagation. A synopsis of these studies demonstrate that supplementation of carotenoids increases the number of circulating lymphocytes (T-helper cells), enhances T and B lymphocyte proliferation, improves rejection of foreign tissue, increases killer cell destruction of tumor cells, enhances neutrophil killing of *Candida* fungi, and inhibits loss of macrophage receptors (Bendich, 1990). Mice fed carotenoids had significantly reduced tumor growth when the primary lesion was excised and then re-challenged with the same tumor (Tomita, 1987). Virus-induced tumors such as murine sarcoma are slowed by carotenoids, as well as adenocarcinoma, squamous cell carcinoma, fibrosarcoma, and chemically induced tumors (Bendich, 1990). These studies present strong evidence that orally administered carotenoids can directly affect the immune responses to cancerous tumors and lead to a lower tumor burden.

Typically, various chemicals are used to induce specific cancers in rats or mice and different dietary supplements are added or left out to test their effects. In rats, the chemical azoxymethane has been used to induce colon cancer and study the effect of anticancer agents. Chemically induced mice also had a significantly reduced incidence of preneoplastic lesions and neoplasms in the bladder when given 50-ppm dietary astaxanthin. The authors suggest that astaxanthin is a possible chemopreventive agent for bladder carcinogenesis and such an effect is partly due to suppression of cell proliferation (Tanaka, 1994). The incidence and multiplicity of neoplasms in the large intestines were significantly smaller after rats were fed astaxanthin. The authors suggest that astaxanthin is a possible chemopreventer of colon, bladder and oral carcinogenesis due to the suppression of tumor cell proliferation (Tanaka, 1995a). Further studies

showed incidences of preneoplastic and neoplasms in the oral cavity of rats were significantly smaller when they received 100-ppm dietary astaxanthin after the carcinogenic chemical induction. In particular, no oral neoplasms developed when rats were fed astaxanthin and the chemical inducer at the same time (Tanaka, 1995b). Mice fed astaxanthin-rich egg yolks developed only one third as many neoplasms and less incidence compared to the control when stomach tumorigenesis was initiated with chemicals (Lee Sang, 1997).

In other studies rats were induced with a chemical to initiate liver cancer (hepatocarcinogenesis), dietary astaxanthin was found to have a significant influence on the reduction in number and size of neoplastic liver lesions (Astorg, 1997a). Astaxanthin has been shown to reduce the carcinogenicity of aflatoxin by inducing enzymes called "CYP1A" and "CYP1A2" which enhance diversion of toxic byproducts towards detoxification pathways (Gradelet, 1997). At dietary levels of 300 ppm, astaxanthin is a strong inducer of CYP1A1 and CYP1A2. (Gradelet, 1996b). In contrast to lycopene or vitamin A, astaxanthin was very efficient in reducing the number and size of liver preneoplastic foci in aflatoxin-induced carcinogenesis (Gradelet, 1998).

Astaxanthin Functions in the Immune System

Singlet oxygen is also cytotoxic to the immune system by virtue of its ability to catalyze production of free radicals. This action can facilitate degradation of macrophage cell membranes resulting in dysfunction and reduced efficiency of phagocytosis (Bendich, 1991). Carotenoids have been shown to enhance both the non-specific and specific immune system and protect cell membranes and cellular DNA from mutation (Bendich A. 1989). Carotenoids have a significant stimulatory effect on the immune system, as seen by the proliferative response of spleen cells and thymocytes during antibody response of mice. Astaxanthin enhances the release of interleukin-1 alpha and tumor necrosis factor alpha in mice greater than canthaxanthin and beta-carotene. The conclusion of one study was that astaxanthin had the best cytokine-inducing activity and may provide an immunomodulating role (Okai, 1996).

In one series of immune system challenges, astaxanthin enhanced T-helper cell antibody production even when suboptimal amounts of antigen were present. Furthermore, astaxanthin, but not other carotenoids (canthaxanthin, beta-carotene, lutein, lycopene), increased the number of antibody-secreting cells from primed spleen cells (Jyonouchi, 1996). Using human blood, it was shown that astaxanthin enhances the production of IgM, IgA and IgG antibodies in response to T-dependent stimuli (Jyonouchi, 1995a and 1995b). Another study indicated a significant immunomodulating action of astaxanthin for humoral immune responses to T-dependent antigens and the authors suggest that carotenoid supplementation may be beneficial in restoring humoral immune responses in older animals. Furthermore, it was speculated that dietary carotenoids could reduce the chance of developing autoimmunity and malignancies by enhancing T-helper functions and promoting specific antibody responses (Jyonouchi, 1994).

References

Astorg P., S. Gradelet, R. Berges, and M. Suschetet. 1997a. Dietary lycopene decreases the initiation of liver preneoplastic foci by diethylnitrosamine in the rat. Nutr. Cancer. 29(1):60-68.

Bendich A. 1989. Carotenoids and the immune response. J. Nutrition 119: 112-115.

Bendich A. 1990. Carotenoids and the immune system. In: Carotenoids Chemistry and Biology. Ed. N.I. Krinsky, Plenum Press, New York.

Bendich A. 1991. Non vitamin A activity of carotenoids: immunoenhancement. Trends in Food Science and Technology. May, pp. 127-129.

Bertram J.S. et al., 1991. Carcinogenesis. 12:671-678

Bertram J.S. 1991. Cancer prevention by retinoids and carotenoids: proposed role of GAP junctional communication. In: Vitamins and Minerals in the Prevention and Treatment of Cancer. M.M. Jacobs, ed. CRC Press, Boca Raton, pp. 31-50.

Britton G., G. M. Armitt, S. Y. M. Lau, A. K. Patel, and C. C. Shone: Carotenoproteins, in "Carotenoid Chemistry & Biochemistry" (ed. by G. Britton and T. W. Goodwin), Pergamon Press, Oxford, 1981, pp. 237-251.

Christiansen R., O. Lie and O.J. Torrissen. 1994. Effect of astaxanthin and vitamin A on growth and survival during first feeding of Atlantic salmon, *Salmo salar*. Aquacult. Fish. Management. 25:903-914.

Christiansen R., O. Lie, and O.J. Torrissen. 1995a. Growth and survival of Atlantic salmon, *Salmo salar* L., fed different dietary levels of astaxanthin. First-feeding fry. Aquaculture Nutrition. 1:189-198.

Christiansen R., J. Glette, O. Lie, O.J. Torrissen and R. Waagbo. 1995b. Antioxidant status and immunity in Atlantic salmon, *Salmo salar* L., fed semi-purified diets with and without astaxanthin supplementation. J. Fish Diseases 18:317-328.

Craik J.C.A. 1985. Egg quality and pigment content in salmonid fishes. Aquaculture 47:61-88.

Challem J. 1998. The comeback carotenoids. Nutrition Science News. 3(4): 192-196.

Davis B.H. Carotenoid metabolism in animals: A biochemist's view: *Pure Appl. Chem.*, 57, 679-684 (1985).

Di Mascio P., M.E. Murphy, and H. Sies. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch. Biochem. Biophys. 274: 532-538.

Di Mascio P.D., M. Murphy, and H. Sies. 1991. Antioxidant defense systems: the role of carotenoids, tocopherols, and thiols. Am. J. Clin. Nutr. 53:194S-200S.

Foote C.S. and R.W. Denny. 1968. Chemistry of singlet oxygen. VIII. Quenching by beta-carotene. J. Am. Chem. Soc. 90:6233-6235.

Foss P., Renstrom B., and S. Liaaen-Jensen. 1987a. Natural occurrence of enatiomeric and meso astaxanthin in crustaceans including zooplankton. Comp. Biochem. Physiol. 86B:313-314.

Goodwin T.W. The Biochemistry of the Carotenoids, 2nd ed., Chapman and Hall, London, 1984, pp. 64-96.

Goodwin. T.W. and M. Jamikorn. 1954. Studies in carotenogenesis. II. Carotenoid synthesis in the alga *Haematococcus pluvialis*. Biochem. J. 57: 376-381.

Grung M., F.M.L. D'Souza, M. Borowitzka, and S. Liaaen-Jensen. 1992. Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S, 3'S)-astaxanthin esters. J. Appl. Phycol. 4: 165-171.

Grung M., Y.S. Svendsen, and S. Liaaen-Jensen. 1993. The carotenoids of eggs of wild and farmed cod. Comp. Biochem. Physiol. 106B:237-242.

Gradelet S., P. Astorg, J. Leclerc, J. Chevalier, M.F. Vernevaut and M.H. Siess. 1996b. Effects of canthaxanthin, lycopene and lutein on liver xenobiotic-metabolizing enzymes in the rat. Xenobiotica. 26:49-63.

Gradelet S., P. Astorg, A.M. Le Bon, R. Berges, and M. Suschetet. 1997. Modulation of aflatoxin B1 carcinogenicity, genotoxicity and metabolism in rat liver by dietary carotenoids: evidence for a protective effect of CYP1A inducers. Cancer Lett. 114(1-2):221-223.

Gradelet S., A.M. Le Bon, R. Berges, M. Suschetet, and P. Astorg. 1998. Dietary carotenoids inhibit aflatoxin B1-induced liver preneoplastic foci and DNA damage in the rat: role of the modulation of aflatoxin B1 metabolism. Carcinogenesis 19(3):403-411.

Haila K.M., B.R. Nielsen, M.I. Heininen, and L.H. Skibsted. 1997. Carotenoid reaction with free radicals in acetone and toluene at different oxygen partial pressures. An ESR spin-trapping study of structure-activity relationships. Zeit. Fuer Leben-Unters. Forsch. 204(2):81-87.

Jorgensen K. and L. Skibsted. 1993. Carotenoid scavenging radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. Z. Lebensm. Unters Forsch. 196:423-429.

Jyonouchi H., L. Zhang, M. Gross, and Y. Tomita. 1994. Immunomodulating actions of carotenoids: enhancement of in vivo and in vitro antibody production to T-dependent antigens.

Jyonouchi H., S. Sun, and M.D. Gross. 1995a. Astaxanthin, a carotenoid without vitamin a activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen. J. Nutr. 125(10):2483-2492.

Jyonouchi H, S. Sun, and G. Myron. 1995b. Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: Astaxanthin, a carotenoid without vitamin A activity, enhances in vitro immunoglobulin production in response to a T-dependent stimulant and antigen. Nutr. Cancer. 23(2):171-183.

Jyonouchi H., S. Sun, M. Mizokami, and M.D. Gross. 1996. Effects of various carotenoids on cloned, effector-stage T-helper cell activity. Nutr. Cancer 26(3):313-324.

King T.J., F. Khachik, H. Bortkiewicz, L. Fukushima, S. Morioka nd J.S. Bertram. 1997. Metabolites of dietary carotenoids as potential preventive agents. Pure and Appl. Chem. 69(10):2135-2140.

Kitahara T. 1984. Carotenoids in the Pacific salmon during the marine period. Comp. Biochem. Physiol. 78B:859-862.

Kobayashi. M. et al. 1992a. Effects of light intensity, light quality, and illumination cycle on astaxanthin formation in green alga, *Haematococcus pluvialis*. J. Ferm. Bioeng. 74(1): 61-63.

Krinsky N.I. 1989. Carotenoids in medicine, In, "Carotenoids. Chemistry and biochemistry". Plenum Press, New York,. M.M. Mathews-Roth and R.F. Taylor. pp. 279-291.

Kurashige M., Okimasu M. and K. Utsumi. 1990. Inhibition of oxidative injury of biological membranes by astaxanthin. Physiol. Chem. Phys. Med. NMR 22(1):27-38.

Lawlor S.M. and O' Brien N.M. 1995. Astaxanthin: Antioxidant effects in chicken embryo fibroblasts. Nutrition Research 15:1695-1740.

Lambertsen C. and O.O. Braekkan. 1971. Method of analysis of astaxanthin and its occurrence in some marine products. J. Sci. Fd. Agric. 22:99-101.

Lee Sang H, W. Park Cherl, S. park Wong, C. Lee Young, S. Choi Eui, and L. Ha Yeong. Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by astaxanthin containing egg yolks. Agric. Chem. Biotechnol. 40(6):490-494.

Lim B.P., A. Nagao, J. Terao, K. Tanaka, T. Suzuki, and K. Takama. Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. Biochim. Biophys. Acta. 1126(2):178-184.

Maoka T., M. Katsuyama, N. Kaneko, and T. Matsuno. 1985. Stereochemical investigation of carotenoids in the antarctic krill *Euphausia superba*. Bull. Jap. Soc. Sci. Fish. 51:1671-1673.

Matsuno T. and S. Hirao. Marine Carotenoids, in "Marine Biogenc Lipids, Fats, and Oils" (ed. by R. G. Ackman), Vol. 1, CRC Press, Florida, 1989, pp. 251-388.

Miki W. 1991. Biological functions and activities of animal carotenoids. Pure and Applied Chem. 63:141-146.

Milon A., G. Wolff, and Y. Nakatani. 1986. Organic carotenoid-phospholipid bilayer systems. Incorporation of zeaxanthin, astaxanthin and their C50 homologous dimyristoylphosphatidylcholine vesicles. Helv. Chemica. 69:12-24.

Muriana F.J.G., V. Ruiz-Gaterrez, M.L. Gallardo, and M.I. Minguez-Mosquera. 1993. A study of the lipids and carotenoprotein in the prawn *Penaeus japonicus*. J. Biochem. 114:223-229.

Murillo E. 1992. Hypercholesterolemic effect of canthaxanthin and astaxanthin in rats. Arch. Latinoam Nutr. 42(4):409-413.

Nakagawa K., S. Kang, D. Park, G. Handelman and T. Miyazawa. 1997. Inhibition by beta-carotene and astaxanthin of NADPH-dependent microsomal phospholipid peroxidation. J. Nutrit. Sci. and Vitamin. 43(3):345-355.

Nishigaki I., A. Dmitrovskii, W. Miki and K. Yagi. 1994. Suppressive effect of astaxanthin on lipid peroxidation induced rats.

Nur-E-Borhan, S. Okada, S. Watabe, and K. Yamaguchi. 1995. Carotenoproteins from the exoskeleton of commercial black tiger prawn. Fish. Sci. 60:213-215.

O'Connor I. and N. O'Brien. 1998. Modulation of UVA light-induced oxidative stress by beta-carotene, lutein and astaxanthin in cultured fibroblasts. J. Dermatol. Sci. 16(3):226-230.

Okai Y. and K. Higashi-Okai. 1996. Possible immunomodulating activities of carotenoids in in vitro cell culture experiments. Int. J. Immunopharmacol. 18(12):753-758.

Oshima S., F. Ojiima, H. Sakamoto, Y. Ishiguro, and J. Terao. 1993. Inhibitory effect of beta-carotene and astaxanthin on photosensitized oxidation of phospholipid bilayers. J. Nutrit. Sci. Vitaminol. 39(6):607-615.

Palozza P. and N.I. Krinski. 1992. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Arch. Biochem. Biophys. 297(2):291-295.

Park J.S., B. P. Chew and T.S. Wong. 1997. FASEB 11:A447, #2586.
Ranby B. and J.F. Rabek, editors. 1978. Singlet Oxygen. Wiley, Chichester, England.

Renstrom B., G. Borch, O. Skulberg, and S. Liaaen-Jensen. 1981. Optical purity of (3S,3'S)-astaxanthin from *Haematococcus pluvialis*. Phytochem. 20(11): 2561-2564.

Renstrom B. and S. Liaaen-Jensen. 1981. Fatty acid composition of some esterified carotenols. Comp. Biochem. Physiol. B., Comp. Biochem. 69: 625-627.

Savoure N., G. Briand, M.C. Amory-Touz, A. Combre, M Maudet and M. Nichol. 1995. Vitamin A status and metabolism of cutaneous polyamines in the hairless mouse after UV irradiation: action of beta-carotene and astaxanthin. Int. J. Vitam. Nutr. Res. 65(2):79-86.

Seddon J.M. et al.. 1994. Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. JAMA 272(18): 1413-1420.

Shimidzu N., M. Goto, and W. Miki. 1996. Carotenoids as singlet oxygen quenchers in marine organisms. Fisheries Science. 62(1): 134-137.

Tacon A.G. 1981. Speculative review of possible carotenoid function in fish. Prog. Fish. Cult. 43(4):205-208.

Tanaka T. Y. Morishita, M. Suzui, T. Kojima, A. Okumura, and H. Mori. 1994. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoids astaxanthin. Carcinogenesis. 15(1):15-19.

Tanaka T., T. Kawamori, M. Ohnishi, H. Makita, H. Mori, K. Satoh, and A. Hara. 1995a. Suppression of azomethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. Carcinogenesis 16(12):2957-2963.

Tanaka T., H. Makita, M. Ohnishi, H. Mori, K. Satoh, and A. Hara. 1995b. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. Cancer Res. 55(18):4059-4064.

Terao J. 1989. Antioxidant activity of beta-carotene related carotenoids in solution. Lipids. 24:659-662.

Tinkler, J.H., F. Bohm, W. Schalch, and T.G. Truscott. 1994. Dietary carotenoids protect human cells from damage. J. Photochem. Photobiol B. 26(3):283-285.

Tomita Y., K. Himeno, K. Nomoto, and H. Endo. 1987. Augmentation of tumor immunity against synergistic tumors in mice by beta-carotene. J. Natl. Cancer Inst. 78: 679-681.

Torrissen O.J. 1990. Biological activities of carotenoids in fishes. The current status of fish nutrients in aquaculture. pp. 387-399. In, Proceedings of the third international symposium on feeding and nutrition in fish. Eds. M. Takeda and T. Watanabe. Tokyo University of Fisheries, Tokyo, Japan.

Truscott T.G. 1990. The photophysics and photochemistry of the carotenoids. J. Photochem. Photobiol. B6: 359-371.

Woodall A., G. Britton and M. Jackson. 1997. Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: Relationship between carotenoid structure and protective ability. Biochim. Biophys. Acta. 1336(3):575-586.

Yamaguchi K., W. Miki, N. Toriu, Y. Kondo, M. Murakami, S. Konsu, M. Satake, and T. Fujita : The Composition carotenoid pigments in the Antarctic Krill *Euphasia superba. Nippon Suisan Gakkaishi.* 49:1411-1415 (1983).

Zhang L.-X., R.V. Cooney, and J.S.Bertram. 1991. Carcinogenesis. 12:2109-2114

Zhang L.X., R.V. Coonery, and J.S. Bartram. 1992. Carotenoids up-regulate Connexin-43 gene expression independent of their provitamin A or antioxidant properties. Cancer Res. 52: 5707-5712.

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Appendix 1

Composition and General Properties of *Haematococcus* algae

Haematococcus algae meal also provides a balanced proportion of vitamins, proteins, fats, carbohydrates, and minerals as 1.0% (10,000 ppm) astaxanthin. The general composition of *Haematococcus* algae consists of common carotenoids, fatty acids, proteins, carbohydrates, and minerals, and is listed in Table 1. Some physical properties are listed in Table 2.

Table 1: Typical Common Components of *Haemtococcus* algae

	Minimum	Maximum	Mean
Protein	17.30	27.16	23.62
Carbohydrates	36.9	40.0	38.0
Fat	7.14	21.22	13.80
Iron (%)	0.14	1.0	0.73
Moisture	3.0	9.00	6.0
Magnesium (%)	0.85	1.4	1.14
Calcium (%)	0.93	3.3	1.58
Biotin (mg/lb.)	0.108	0.665	0.337
L-carnitine (ug/g)	7.0	12	7.5
Folic acid (mg/100g)	0.936	1.48	1.30
Niacin (mg/lb.)	20.2	35.2	29.8
Pantothenic acid (mg/lb.)	2.80	10.57	6.14
Vitamin B1 (mg/lb.)	<0.050	4.81	2.17
Vitamin B2 (mg/lb.)	5.17	9.36	7.67
Vitamin B6 (mg/lb.)	0.659	4.5	1.63
Vitamin B12 (mg/lb.)	0.381	0.912	0.549
vitamin C (mg/lb.)	6.42	82.7	38.86
Vitamin E (IU/lb.)	58.4	333	186.1
Ash	11.07	24.47	17.71

Table 2: Physical Characteristics *Haematococcus* algae:

Color	Red to Dark red
Particle size	5-25 microns
Moisture	4-9%
Bulk density	
loose value	0.303-0.345 g/ml
tapped value	0.370-0.435 g/ml
Astaxanthin	1.5 & 2.0%

The amino acid profile of *Haematococcus* algae is listed in Table 3.

Table 3: Typical Amino Acid Analysis of *Haematococcus* algae

	Minimum value	Maximum value	Mean
Tryptophan	0.05	0.56	0.31
Aspartic acid	1.37	2.31	1.89
Threonine	0.78	1.24	1.04
Serine	0.73	1.06	0.94
Glutamic acid	1.70	2.39	2.19
Proline	0.69	1.00	0.89
Glycine	0.84	1.32	1.17
Alanine	1.30	1.92	1.73
Cysteine	0.16	0.21	0.19
Valine	0.83	1.94	1.36
Methionine	0.32	0.43	0.40
Isoleucine	0.55	0.97	0.79
Leucine	1.21	1.84	1.67
Tyrosine	0.40	0.63	0.52
Phenylalanine	0.61	1.05	0.90
Histidine	0.48	0.76	0.61
Lysine	0.75	1.32	1.13
Arginine	0.81	1.34	1.07

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Bringing the Ocean to Life

AstaFactor® Technical Report

Haematococcus Pluvialis and Astaxanthin Safety For Human Consumption

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Safety for human consumption of *Haematococcus pluvialis* algal meal and astaxanthin has been demonstrated by a number of studies:

- **A recent 28-day rat study with *Haematococcus pluvialis* dry algal meal, produced by Mera Pharmaceuticals' proprietary technology, demonstrated that there was no observed sub-acute toxicity at a daily dose of 50 mg/kg body weight, corresponding to 3,500 mg algal meal per 70-kg body weight of a typical adult man.**

- **No lethality was seen for *Haematococcus pluvialis* algae at doses up to 5000 mg/kg body weight, in an earlier, 13-day, single-dose (acute-toxicity), rat study.**

- **A human safety study demonstrated that daily ingestion of up to 1,140 mg Mera Pharmaceuticals's *Haematococcus pluvialis* algal meal, for 29 days, did not result in any safety concern.**

- **A recent sub-acute rat toxicity with Mera Pharmaceuticals' *Haematococcus pluvialis* algal meal, showed no signs of toxicity, after dosing rats with up to 1.15 mg astaxanthin per kg body weight per day (equivalent to 80.5 mg astaxanthin per 70-kg body weight) for 28 consecutive days.**

- **In a human safety study with Mera Pharmaceuticals' algal astaxanthin, no sign of toxicity or safety concern was observed, when volunteers ingested up to 19.25 mg astaxanthin per day for 29 days, while an earlier human study failed to find any harmful effect from 14.4 mg/day astaxanthin ingestion for two weeks.**

- **Pure astaxanthin (up to 80 mg/kg feed), is Generally Considered As Safe by FDA, for use in salmon diets. This can result in astaxanthin deposition of 10 to 15 mg/kg in salmon fillets. Levels of astaxanthin naturally occurring in wild-caught seafood, and dietary studies on carotenoids, seafood, and salmon, also suggest that a daily serving of 5 mg astaxanthin, corresponding to 125 g of wild-caught Sockeye salmon fillet or less than 100 g of krill, is safe.**

- **The proprietary technology and quality control developed by Mera Pharmaceuticals to produce *Haematococcus pluvialis* algal meal, ensure that the**

product meets dietary supplement safety standards.

Conclusion: A supplement containing 5 mg astaxanthin derived from 250 mg, or less, of Mera Pharmaceuticals' *Haematococcus pluvialis* algal meal is safe for daily human consumption.

Mera Pharmaceuticals' proprietary technology allows the production of a high quality algal meal containing 2% total astaxanthin or more. It is therefore a very good source of natural astaxanthin, a carotenoid pigment and biological antioxidant widely encountered in nature. Safety for human consumption of astaxanthin and ***Haematococcus pluvialis*** algae has been demonstrated by a number of studies.

1. Toxicity studies

1.1. *Haematococcus* algae.

1.1.1. Human safety study

In a recent clinical safety study with Mera Pharmaceuticals's ***Haematocccus pluvialis*** algal meal, 33 human volunteers (15 males and 18 females, age 28 to 62) ingested on a daily basis, for 29 consecutive days, either a Low Dose supplement containing 228 mg algal meal and 3.85 mg astaxanthin, or a High Dose supplement containing 1140 mg algal meal and 19.25 mg astaxanthin.[1]

Volunteers underwent a complete medical examination before, during and at the end of the study. The physician, examined specifically, but not exclusively, the weight, skin coloration, general appearance, blood pressure, vision and eye, (near and distant vision, color vision, depth perception, eye condition), ears and nose, mouth, throat and teeth, chest and lungs, and reflexes, for each volunteer.

This medical examination was complemented by extensive urine analyses and blood analyses (cell counts, hemoglobin, liver enzyme activity indicators, and other blood parameters) (Table 1). No ill effects or toxicity from ingestion of the supplement were observed, confirming the absence of toxicity of Mera Pharmaceuticals' ***Haematococcus pluvialis*** algal meal.

1.1.2. Rat toxicity studies

Absence of toxicity of ***Haematococcus pluvialis*** has also been demonstrated in rats and mice, widely accepted animal models for safety assessment of human dietary supplements.

A 28-day sub-acute rat toxicity study, with ***Haematococcus pluvialis*** algal meal produced with Mera Pharmaceuticals' proprietary technology, failed to find any sign of toxicity of this algal meal.[2]Three groups of 20 rats each (10 males/10 females) were fed daily by gavage 0, 5, or 50 mg/kg algal meal in a corn oil suspension for 28 consecutive days (corresponding to daily doses of 0, 350 mg and 3,500 mg algal for 70-kg body weight). After sacrifice, the

post-mortem observations, hematology and clinical chemistry failed to detect any sign of toxicity.

An earlier 13-day rat toxicity study demonstrated that the LD50 acute toxicity of ***Haematococcus pluvialis*** algal meal in rats was greater than 5000 mg/kg.[3] In this study, three separate groups of 10 rats (5 males and 5 females per group) were fed 5,000 mg/kg algal meal suspended in a 0.5% methylcellulose solution. Mortality, body weights, necropsy examination and pharmacotoxic signs were evaluated on each group. The study found no remarkable differences in body weights or visible abnormalities. The post-mortem examination after sacrificing the animals at the end of the study revealed no abnormalities.

Another acute toxicity trial was reported with male and female mice.[4] In this study, ***Haematococcus pluvialis*** algal meal was suspended in distilled water for gavage to give a 30% solution (w/v). The solution was given in a single dose, at dosages ranging from 10,417 to 18,000 mg/kg. No mortalities occurred and no abnormalities were observed in the post-mortem examination. When converted to a 70-kg body weight, these doses are equivalent to single doses ranging from 729 g to 1,260 g.

1.1.3. Other studies

In salmonids, numerous experiments have shown that ***Haematococcus pluvialis*** can be incorporated in the diet at dosages ranging from 0.1% to 6% without any negative effect on growth or survival.[5,6,7,8] A recent report showed no indication of disease, toxicity or neoplasia in fish fed ***Haematococcus pluvialis*** as a dietary source of astaxanthin.[4] The fish were reported in excellent nutritional status with abundant body fat. Studies have also indicated that feeding ***Haematococcus pluvialis*** can enhance growth and/or survival in trout and shrimp.[8-10]

1.2. Astaxanthin.

Astaxanthin naturally appears in the human diet when seafood such as salmon, red fishes, shrimp, krill or lobster are eaten.

1.2.1. Human studies

The recent clinical safety study, mentioned above, proved the safety of astaxanthin from Mera Pharmaceuticals' ***Haematocccus pluvialis*** algal meal. [1] In that study, 33 human volunteers (15 males and 18 females, age 28 to 62) ingested on a daily basis, for 29 consecutive days, either 3.85 mg or 19.25 mg algal astaxanthin. As mentioned earlier, extensive blood and urine analyses were conducted throughout the study (Table 1), and the physician conducted a detailed medical examination. Based on the results of these urine and blood analyses and the observations of the physician, no sign of toxicity from astaxanthin was detected even at the higher dose.

In a study with healthy human patients, who ingested up to 14.4 mg/day astaxanthin for two weeks, no ill effect was reported.[11] On the contrary, a positive antioxidant effect of

astaxanthin on serum Low Density Lipoprotein (LDL) was observed. In that study, thirteen healthy patients were selected, subdivided into 3 groups, and given three levels of astaxanthin daily, for two weeks, as follows: 5 patients fed 3.6 mg/day, 5 patients fed 7.2 mg/day, and 3 patients fed 14.4 mg/day. The astaxanthin was administered sublingualy in the form of a softgel capsule. Blood samples were taken and the LDL fraction was collected and exposed to an oxidizing agent. The study demonstrated that increasing doses of astaxanthin significantly and increasingly slowed down the oxidation of the LDL fraction.

1.2.2. Rat toxicity studies

In the recent study with Mera Pharmaceuticals' ***Haematococcus pluvialis*** algal meal, described above, rats ingested daily up to 1.15 mg astaxanthin per kg body weight (equivalent to 80.5 mg for 70-kg body weight per day), for 28 days, without showing any sub-acute toxicity sign.

Other animal studies on the effects of astaxanthin have shown that even higher doses could be fed to rats for prolonged periods. Some of these studies have demonstrated beneficial results. In one study, feeding rats 500 ppm astaxanthin for 34 consecutive weeks resulted in reduced cancer occurrence in the intestinal and oral mucosa and improved the condition of the oral cavity.[12,13]

1.2.3. Safety of astaxanthin in food salmon – safe daily dose of astaxanthin

For years, astaxanthin has been added to aquaculture diets at levels of up to 200 mg/kg, without any toxic effect on target animals. Additionally, numerous studies have demonstrated improved growth, survival and immune response in fish and shrimp.[8-10,14-23] Astaxanthin is regularly added at 50 ppm or higher to commercial diets fed to food fish for prolonged periods, i. e., for up to 2 years in the case of farmed salmon.

According to the Code of Federal Regulations, astaxanthin is Generally Recognized As Safe ("GRAS") when used as a color additive in salmon foods, with a maximum inclusion of 80 mg/kg feed.[24] Numerous studies have shown that such an inclusion level results in accumulation of astaxanthin in the flesh of Atlantic salmon at levels between 4 and 10 mg/kg, and at even higher levels in other species (Table 2).

These levels in Atlantic salmon are comparable to or slightly higher than levels observed in their wild counterparts, but lower than levels found in other wild salmon species found on the Pacific coast of the United States, where values as high as 58 ppm in Sockeye salmon were reported by a recent FDA study.[25] (Average of astaxanthin measurements in this study were 13.8 ppm in Coho salmon and 40.4 ppm in Sockeye salmon).

It was noted that the main astaxanthin stereo isomer identified by the FDA researchers in the 5 species of wild Pacific salmon they studied, was the 3S,3'S stereo isomer, identical to that found in ***Haematococcus pluvialis***.[8,25]

Salmon, a fish rich in omega-3 fatty acids, is considered a healthy food, and, like other

sources of these poly-unsaturated fatty acids, is highly recommended by nutritionists.[26-29] According to an epidemiological study on Alaska's native and non-native residents, the lowest rate of ischaemic heart disease mortality, less than one-third that of US Caucasians, occurred in Alaskan Eskimos who lived in an area with documented patterns of high salmon consumption by individuals with high blood concentrations of omega-3 fatty acids.[28] Based on the salmon flesh astaxanthin values mentioned above, a daily consumption of a 200-g portion of wild Sockeye salmon with 40 ppm astaxanthin in the flesh would lead to a daily ingestion of 8 mg astaxanthin per day. From a different point of view, the intake of a 5 mg supplement of astaxanthin corresponds to eating 500 g per day of farmed rainbow trout or Atlantic salmon, 125 g of wild Sockeye salmon, or less than 100 g of krill.

Based on these published data, as well as the animal toxicity data publicly available, it may be inferred that the ingestion of 5 mg astaxanthin per day by an adult human is reasonably safe. This was further substantiated by Mera Pharmaceuticals' 29-day human safety study, which investigated the safety of 3.8 mg astaxanthin/day and 19 mg/day astaxanthin from ***Haematococcus pluvialis*** algal meal, i. e., almost four-fold higher than the assumed safe daily dose of 5 mg.[1]

The results of the extensive blood and urine analyses and complete physical examinations before, during, and at the end of the trial period, raised no apparent safety concern. The data were reviewed by two independent physicians, a clinical pathologist and a professional pharmacotoxicologist, all of who concurred that both doses were safe.

2. Non-mutagenicity of *Haematococcus*

A recent study[30] reported no mutagenic effect of ***Haematococcus pluvialis*** algae, using a mutagenicity test with *Salmonella typhimurium* strains TA100, TA1535, TA98, TA1537, TA1538, and E.coli WP2 uvr A.

In this experiment, ***Haematococcus pluvialis*** algal meal was formulated in a 50mg/mL solution of dimethyl sulfoxide. The formulation was spread onto petri dishes in the presence of the microbial cultures with positive controls. The positive controls (mutagenic agents): 2-(2-furyl)-3-5(5-nitro-2-furyl)acrylamide, 1-ethyl-2-nitro-3-nitrosoguanidine, 9-aminoacridine, 2-aminoanthracene, and 2-nitrofluorene, showed a remarkable increase in the number of reverent colonies in every case, compared to the solvent control.

3. Carcinogenicity

Haematococcus pluvialis is not known to have any carcinogenic effect, or contain significant levels of recognized carcinogens. On the contrary, ***Haematococcus pluvialis*** contains a high level of astaxanthin which has widely demonstrated anticarcinogenic effects.[31-35]

4. Heavy metals

Haematococcus pluvialis algae produced and processed by Mera Pharmaceuticals for human food consumption meet the Federal Food and drug Administration's list of maximum

tolerances:

- Heavy metals (as lead): < 10.0 ppm
- Mercury < 1.0 ppm
- Cadmium < 0.5 ppm
- Arsenic < 2.0 ppm
- Lead < 5.0 ppm

This has been confirmed by analyses of various batches (Lot HP980051[36] and Lot 990610Mix[37], a blend resulting from combining five batches: Lots 990513A, 990518B, 990520A, 990524A, 990526A, and therefore, highly representative of the quality of ***Haematococcus pluvialis*** algal meal produced with Mera Pharmaceuticals' technology).

5. Bacteriology

Manufacturing process follows FDA GMP recommendations for food supplements to avoid spoilage and contamination of ***Haematococcus pluvialis*** algal meal by harmful micro-organisms or other types of contaminants.

During the processing, the algal biomass is mechanically cell-broken to ensure a thorough rupture of cell walls, undergoes a pasteurization process, and is dried to a moisture content less than 5%. The pasteurization treatment ensures that the following bacteriological specifications in the final product are achieved, as confirmed by analyses by an independent laboratory[37]:

- Total aerobic plate count <1,000 CFU
- Total coliforms <10/g
- ***E. coli*** <10/g
- ***Salmonella*** absence in 25 g

6. Other natural toxic compounds and toxicity risks

Mera Pharmaceuticals is not aware of any significant or detectable levels of known carcinogenic or toxic compounds in ***Haematococcus pluvialis*** algae that could have a negative effect on human health.

Analyses on the algae meal have demonstrated absence of mycotoxins, and especially of aflatoxins.[36,37]

Haematococcus pluvialis may contain small amounts of canthaxanthin, a carotenoid pigment closely related to astaxanthin. Analyses have shown that canthaxanthin concentrations in ***Haematococcus pluvialis*** algal meal produced with Mera Pharmaceuticals proprietary technology are less than 2% of total astaxanthin concentration. Mera Pharmaceuticals' proprietary technology maximizes astaxanthin biosynthesis by Haematococcus pluvialis and in so doing also minimizes the relative proportion of other carotenoids (including canthaxanthin).

At the levels of canthaxanthin encountered in Mera Pharmaceuticals' algal meal, a daily dose of 5 mg algal astaxanthin as a supplement would entail also ingesting 0.1 mg canthaxanthin per day. Although canthaxanthin has been demonstrated to have positive metabolic effects such as an anticancer activity,[38] there has been reports that, at high doses for prolonged periods, it can have negative effects. One case of aplastic anemia associated with canthaxanthin ingested for tanning purposes, was reported a few years ago[39]. Others have reported the appearance of crystalline formations in the retina of some individuals who ingested up to 66 g cantaxanthin over 24 months (corresponding to an average daily ingestion of 90 mg cantaxanthin per day) for tanning purposes[40]. However, later it was demonstrated that these canthaxanthin deposits in the retina could be reversed[39] In any case, the levels of canthaxanthin that would be ingested through a 5 mg astaxanthin dietary supplement formulated with Mera Pharmaceuticals' algal meal are nearly 1000-fold lower than the doses which were observed to cause canthaxanthin maculopathy. Therefore, they should represent no safety risk.

The rat toxicity and human studies which were conducted with Mera Pharmaceuticals' algal meal confirmed this. It should also be noted that FDA has approved canthaxanthin as a color additive in fish foods (up to 80 mg/kg feed, which can result in canthaxanthin deposition levels of 4 to 12 mg/kg fillet) and broiler diets, as well as in foods and drugs.[41] In foods, the limit authorized by FDA is 30 mg canthaxanthin per pound of solid food. The ingestion of 0.1 mg cantaxanthin in a dietary supplement containing 5 mg astaxanthin, is therefore well below the levels that would be encountered in foods that are considered safe by FDA.

7. Product specifications

A detailed description of the manufacturing process and of the specifications of ***Haematococcus pluvialis*** for use in dietary supplements are reviewed in a separate technical report.[42]

8. Metabolic effects of astaxanthin

Astaxanthin is a powerful natural antioxidant. There is a growing amount of scientific evidence not only on the safety of astaxanthin for human consumption, but on the positive metabolic effects that it may have. These findings have been reviewed in detail in Mera Pharmaceuticals Technical Reports TR.3002.001[43] and TR.3003.001 [44].

9. Dietary studies - safe daily dose of algal astaxanthin

Astaxanthin appears to be absorbed in the blood in the same way as other carotenoids. Carotenoids are absorbed by passive diffusion through the intestinal mucosa after being emulsified and solubilized in lipid micelles which are incorporated into chylomicrons when exiting the intestinal mucosal cells.[45] They are transported in the blood after being transferred from the chylomicrons to lipoproteins.

In a recent human study, a single dose of 100 mg dietary astaxanthin was not found to have

any negative effect and demonstrated that astaxanthin has a similar absorption pattern to other carotenoids.[46] Astaxanthin was measured in the blood plasma of 3 middle-aged male subjects after ingestion of a single dose of 100 mg astaxanthin. Astaxanthin was readily absorbed and transported by various lipoproteins: chylomicrons/Very Low Density Lipoproteins, High Density Lipoproteins and Low Density Lipoproteins.

Plasma levels of astaxanthin peaked at 1.2 mg/L (= 2 μmol/L) after 6 hours and progressively declined over the next 66 hours to a 0.2 mg/L level. These levels and duration are comparable to levels reported in the literature for other carotenoids.[47-49] Astaxanthin appears to be absorbed at a similar rate than beta-carotene which peaks in the serum after 6 to 9 h.[49] In mice, astaxanthin also appeared to be absorbed quite effectively, when compared to beta-carotene or lutein.[49]

The official recommended dietary intake for vitamin A is 1,000 retinol equivalents, for men, and 800 for women.[51] This corresponds to 6 μg (***micro***grams) beta-carotene or 12 μg of other pro-vitamin A carotenoids.[51] On the other hand, practical levels of carotenoid intake are significantly higher. Epidemiological studies in North Europe have found daily ingestion of carotenoids ranging from 2.9 to 7.6 mg/ (***milli***grams) per day,[52-54] while in the US, the level of carotenoids supplied by the "normal" diet is estimated to be 1.5 mg beta-carotene per day.[51]

The Alliance for Aging Research, a US Citizen Advocacy organization for research to improve the health and independence of older people, has recommended 10 to 30 mg beta-carotene per day for optimal health, and doses of 20 to 180 mg beta-carotene for many years have been used to treat erythropoietic protoporphyria, with no evidence of toxicity and without development of abnormally-elevated blood vitamin A levels.[51] In addition it should be noted that astaxanthin, unlike other carotenoids such as beta-carotene, has no provitamin A activity;[55,56] therefore it represents a lower risk of hyper-vitaminosis A.

It may be argued that because astaxanthin is closely related to canthaxanthin it could also have similar toxic effects as those described above. However, the available data indicate that astaxanthin consumption at no greater than the recommended dose of 5 mg per day poses no safety risk:

- The proposed daily intake of astaxanthin (5 mg) is much lower than the levels of canthaxanthin which were found to have toxic effects (up to 90 mg average daily intake for 24 months).
- The human safety study conducted with Mera Pharmaceuticals' algal astaxanthin found no changes in vision or eye condition in the patients. Another good indicator, skin coloration, did not change throughout the Mera Pharmaceuticals safety study.
- The post-mortem examination of the animals in Mera Pharmaceuticals' rat toxicity study also failed to find any adverse effect of astaxanthin supplementation at the doses tested.

- Researchers at the University of Illinois also reported that, in an animal model (rats), astaxanthin, unlike canthaxanthin, did not form crystalline depositions in the eye. [57] Furthermore, they demonstrated that astaxanthin can have a beneficial role in the protection of the eyes from UV-light damage.

In conclusion, based on published studies (reviewed above), on natural levels of astaxanthin found in seafood, and on the results of the studies conducted by Mera Pharmaceuticals, it appears that the consumption by a healthy adult human of a daily dose of 5 mg astaxanthin, in the form of a supplement formulated with 250 mg (or less) ***Haematococcus pluvialis*** algal meal produced with Mera Pharmaceuticals' proprietary technology, represents no safety risk. This suggested dose is approximately four times lower than the high dose which was demonstrated to be safe by Mera Pharmaceuticals' safety study.

References

1. Mera Pharmaceuticals Inc. 1999. A human safety trial of natural astaxanthin from Haematococcus pluvialis algae, produced with Mera Pharmaceuticals' proprietary technology. Confidential Report: RD.0100.001.

2. MB research laboratories. 1999. 28 day repeated dose oral toxicity study in rates. Confidential report to Mera Pharmaceuticals Inc.

3. International Research and Development Corporation. 1989. Acute oral toxicity Study in Rats for Microbio Resources Inc. Appendix 23, in: FDA website Docket Number 95S-0316, Report #45, (URL: http://www.fda.gov/ohrms/dockets /dailys/062199/rpt00045.htm). Access date: November 27, 1999.

4. Koyo Mercantile Company ltd. 1988. Acute Toxicity Test for Riken Vitamin K.K. Appendix 24, in FDA website, Docket Number 95S-0316, Report #45, (URL: http://www.fda.gov/ohrms/dockets /dailys/062199/rpt00045.htm). Access date: November 27, 1999.

5. Spitsbergen J. 1997. Gross pathologic examination of salmonids from dietary study with Natural Astaxanthin (***Haematococcus*** algae meal). Appendix 26, in FDA website Docket Number 95S-0316, Report #45, (URL: http://www.fda.gov/ohrms/dockets /dailys/062199/rpt00045.htm). Access date: November 27, 1999.

6. Sommer T.R., F.M.L.D. Souza and N.M. Morissy. 1991. Pigmentation of adult rainbow trout, Onchorhynchus mykiss, using the green alga ***Haematococcus pluvialis***. Aquaculture, 106, 63-74.

7. Choubert G. and O. Heinrich. 1993. Carotenoid pigments of the green ***alga Haematococcus pluvialis*** : assay on rainbow trout ***Onchorhynchus mykiss***, pigmentation in comparison with synthetic astaxanthin and cantaxanthin. Aquaculture 112, 217-226.

8. Mera Pharmaceuticals Inc. 1999. Technical report TR2102.001: Aquaxan™ HD algae meal use in aquaculture diets: Enhancing nutritional performance and pigmentation.

9. Darachai J., S. Piyatiratitivorakul, P. Kittakoop, C. Nitithamyong, P. Menasveta. 1998. Effects of astaxanthin on larval growth and survival of the giant tiger prawn, Penaeus monodon. the Vth Asian Fisheries Forum in Chiang Mai, Thailand (November 11-13, 1998).

10. Christiansen R., O. Lie, O.J. Torissen. 1995. Growth and survival of Atlantic ***salmon Salmo salar*** L. fed different dietary levels of astaxanthin. First-feeding fry. Aquac., Nutr. 1, 189-198.

11. Miki, W., K. Hosada, K. Kondo, and H. Itakura. 1998. Astaxanthin-containing drink. Japanese Patent #10155459 [in Japanese].

12. Tanaka, T., T. Kawamori, M. Ohnishi, H. Makita, H. Mori, K. Satoh, and A. Hara. 1995a. Suppression of azomethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. Carcinogenesis 16: 2957-2963.

13. Tanaka, T., H. Makita, M. Ohnishi, H. Mori, K. Satoh, and A. Hara. 1995b. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. Cancer Res. 55: 4059-4064.

14. Christiansen R., O. Lie, and O.J. Torissen. 1994. Effect of astaxanthin and vitamin A on growth and survival of Atlantic salmon fry, ***Salmo salar*** L. Aquac., Fish. Manag. 25:903-914.

15. Verlhac V., J. Gabaudan, and J. Schierle. 1995. In-vitro anti-oxidant properties of astaxanthin on rainbow trout immune cells. In: Developmental and comparative immunology. Clem, L.W. Warr G.W. (Eds.). The VIth ISDC congress. Abstracts. P889. Presented at the Nordic Symposium on Fish Immunology. May 1995, Reykiavik, Iceland.

16. Verlhac V., Gabaudan J., Schierle J. 1995. Influence of astaxanthin on non-specific immune response of rainbow trout. Presented at the Nordic Symposium on Fish Immunology. May 1995, Reykiavik, Iceland. Clem, L.W. Warr G.W. (Eds.). The VIth ISDC congress. Abstracts. Presented at the Nordic Symposium on Fish Immunology. May 1995, Reykiavik, Iceland.

17. Chien Y.H., S.C. Jeng. 1992. Pigmentation of Kuruma prawn, ***Penaeus japonicus*** Bates, by various pigment sources and levels and feeding regimes. Aquaculture, 102, 333-346.

18. Tanaka Y., H. Matsuguchi, T. Katayama, K.L. Simpson, C.O. Chichester. 1976. The biosynthesis of astaxanthin. XVIII. The metabolism of the carotenoids in the prawn, ***Penaeus japonicus*** Bates. Bull. Jpn. Soc. Sci. Fish., 42:197-202.

19. Menasveta P. 1995. Role of micro-nutrients in increasing disease resistance in shrimp. 2nd. Roche Aquaculture Centre Conference on Shrimp Nutrition and Disease. June 15, 1995, Bangkok, Thailand, Ed. K. Kurmali.

20. Nègre-Sadargues G., R. Castillo, H. Petit, S. Sance, R.G. Martinez, J-C.G. Choubert, J-P. Trilles. 1993. Utilisation of synthetic carotenoids by the prawn ***Penaus japonicus*** reared under laboratory conditions. Aquaculture 110, 151-159.

21. Thongrod S., A. Tansutapanich, O.J. Torissen. 1995. Effect of dietary astaxanthin and supplementation on accumulation, survival, and growth in post-larvae of ***Penaeus monodon*** Fabricius. In P.Lavens, E. Jaspers and I. Roelants (eds.). Larvi'95 – Fish & Shellfish Larviculture Symposium. European Aquaculture Society, Special Publication, No. 24, Gent, Belgium, 251-254.

22. Kurmali K. 1995. Shrimp Nutrition and diseae: role of vitamins and astaxanthin. 2nd. Roche Aquaculture Centre Conference on Shrimp Nutrition and Disease. June 15, 1995, Bangkok, Thailand. Ed. K. Kurmali.

23. Chien Y.H. 1996. Biological effects of astaxanthin in shrimp, a review. 3rd Roche conference on nutrition and disease. December 12, 1996, Bangkok, Thailand, Ed. B. Hunter, 73-81.

24. Code of Federal Regulations, Title 21, Volume 1, Parts 1 to 99, Revised as of April 1, 1999, via GPO Access[CITE: 21CFR73], Page 332-377, Part 73: Listing of color additives exempt from certification, Subpart A: Foods, Section 73-35: astaxanthin, Access date: November 27, 1999. URL: http://frwebgate5.access.gpo.gov/cgi-bin/waisgate.cgi?WAISdocID=4096121134+2+0+0&WAISaction=retrieve

25. Turujman S.A., W. G. Wamer, R.R. Wei, R.H. Albert. 1997. Rapid liquid chromatographic method to distinguish wild salmon from aquacultured salmon fed synthetic astaxanthin. J. AOAC Int., 80(3): 622-632.

26. Kromhout D., E.J. Feskens, C.H. 1995. The protective effect of a small amount of fish on coronary heart disease mortality in an elderly population. Int. J. Epidemiol. Apr;24 (2):340-5.

27. Keli S.O., E.J. Feskens, D. Kromhout. 1994. Fish consumption and risk of stroke. The Zutphen Study. Stroke, 25(2):328-32.

28. Davidson M., L.R. Bulkow, B.G. Gellin. 1993. Cardiac mortality in Alaska's indigenous and non-Native residents. Int. J. Epidemiol.;22(1):62-71.

29. Horrocks L.A., Y.K. Yeo. 1999. Health benefits of docosahexaenoic acid. Pharmacol. Res.;40(3):211-25.

30. Cyanotech Corp. 1999. Haematococcus algae safety summary. FDA website Docket Number 95S-0316, Report #45, (URL: http://www.fda.gov/ohrms/dockets /dailys/062199/rpt00045.pdf)062199.htm). Access

date: November 27, 1999.

31. Gradelet, S., P. Astorg, J. LeClerc, J. Chevalier, M.-F. Vernevaut, and M.-H. Siess. 1996. Effects of canthaxanthin, astaxanthin, lycopene and lutein on liver xenobiotic-metabolizing enzymes in the rat. Xenobiotica 26: 49-63.

32. Jyonouchi, H., R.J. Hill, Y. Tomita, and R. A. Good. 1991. Studies of immunomodulating actions of carotenoids. I. Effects of beta-carotene and astaxanthin on murine lymphocyte functions and cell surface marker expression in in vitro culture system. Nutr. Cancer 16: 93-105.

33. Jyonouchi, H., L. Zhang, and Y. Tomita. 1993. Studies of immunomodulating actions of carotenoids. II. Astaxanthin enhances in vitro antibody production to T-dependent antigens without facilitating polyclonal B-cell activation. Nutr. Cancer 19: 269-280.

34. Jyonouchi, H., L. Zhang, M. Gross, and Y. Tomita. 1994. Immunomodulating actions of carotenoids: Enhancement of in vivo and in vitro antibody production to T-dependent antigens. Nutr. Cancer 21: 47-58.

35. Tomita, Y., H. Jyonouchi, R.W. Engelman, N.K. Day, and R.A. Good. 1993. Preventive action of carotenoids on the development of lymphadenopathy and proteinuria in MRL-lpr/lpr mice. Autoimmunity 16: 95-102.

36. Aspland and James Ltd. 1998. Certificate of analysis. Algal meal Lot # HP980051.

37. Warren Analytical laboratory. 1999. Laboratory Analysis Report on Lot # 990610 Mix ***H.pluvialis*** flakes. Confidential report to Mera Pharmaceuticals Inc.

38. Gerster H. 1993. Anticarcinogenic effect of common carotenoids. Int. J. Vitam. Nutr. Res., 63(2):93-121.

39. Leyon H., A.M. Ros, S. Nyberg, P. Algvere. 1990. Reversibility of cantaxanthin deposits within the retina. Acta. Ophthalmol. (Copenh.), 68(5):607-611.

40. Boudreault G., P. Cortin, L.A. Corriveau, A.P. Rousseau, Y. Tardif, M. Malenfant. 1983. Canthaxanthine retinopathy: 1. Clinical study in 51 consumers. Can. J. Opthalmol., 18(7):325-328.

41. U.S. F.D.A. 1999. Section 73.75: Canthaxanthin. Code of Federal Regulations, 21(1) 73: 337-338.

42. Mera Pharmaceuticals Inc. 1999. Technical report TR3004.001. Haematococcus pluvialis algal meal produced with Mera Pharmaceuticals' proprietary technology: a unique source of natural astaxanthin and algal nutrients.

43. Mera Pharmaceuticals Inc. 1999. Technical report TR.3002.001. Astaxanthin as an antioxidant: a summary.

44. Mera Pharmaceuticals Inc. 1999. Technical report TR.3003.001. Astaxanthin and health: a summary.

45. Furr H. C. and R.M. Clark. 1997. Intestinal absorption and tissue distribution of carotenoids. Nutritional Biochem. 8:364-377.

46. Osterlie M., B. Bjerkeng, S. Liaaen-Jensen. 1999. Pigments in food technology, Proc, 1st. Int. Congr. PFT., March 24-26, Sevilla, Spain.

47. Kostic D., W.S. White, J.A. Olson. 1995. Intestinal absorption, serum clearance, and interactions between lutein and beta-carotene when administered to human adults in separate or combined oral doses. Am. J. Clin. Nutr., 62: 604-610.

48. Clark R.M., L. Yao, L. She, H.C. Furr. 1998. A comparison of lycopene and canthaxanthin absorption: using the rat to study the absorption of non-provitamin A carotenoids. Lipids, 33(2):159-63.

49. Gärtner C., W. Stahl, et al. 1996. Preferential increase in chylomicron levels of the xantophylls lutein and zeaxanthin compared to beta-carotene in the human. Int. J. for Vit. and Nutr. Res., 66:119-125.

50. Park JS, BP Chew, TS Wong, JX Zhang, NS Magnuson. 1999. Dietary lutein but not astaxanthin or beta-carotene increases pim-1 gene expression in murine lymphocytes. Nutr. Cancer 33(2):206-212.

51. VERIS. 1996. Carotenoids – Fact Book. Ed. VERIS, La Grange, IL., USA, 32pp.

52. Golbohm R.A., H.A. Brants, K.F. Hulshof, PA van den Brandt. 1998. The contribution of various foods to intake of vitamin A and carotenoids in the Netherlands. Int. J. Vitam. Nutr. Res. 68(6):373-383.

53. Heinonen M. 1991. Food groups as sources of retinoids, carotenoids and Vitamin A in Finland. Internat. J. Vit. Nutr. Res. 61:3-9.

54. Faure H., V. Fayol, C. Galabert, P. Grolier, GL Moel, J. Stephens, F. Nabet. 1999. Carotenoids: 2. Diseases and supplementation studies. Ann Biol. Clin. (Paris), 57 (3):273-282.

55. Jyonouchi, H., S. Sun, and M. Gross. 1995. Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: Astaxanthin, a carotenoid without vitamin A activity, enhances in vitro immunoglobulin production in response to a T-dependent stimulant and antigen. Nutr. Cancer 23:171-183.

56. Jyonouchi, H., S. Sun, Y. Tomita, and M.D. Gross. 1995. Astaxanthin, a carotenoid without vitamin A activity, augments antibody responses in cultures including T-helper cell clones and sub-optimal doses of antigen. J. Nutr. 124:2483-2492.

57. Tso, M.O.M., and T.-T. Lam. 1996. Method of retarding and ameliorating central nervous system and eye damage. U.S. Patent #5527533.

58. Goralczyck R., S. Buser, J. bausch, W. Bee, U. Zuhlke, F.M. Barker. 1997. Occurrence of birefringent retinal inclusions in cynomolgus monkeys after high doses of cantaxanthin. Invest. Ophthalmol. Vis. Sci., 38(3):741-752.

59. Leyon H., A.M. Ros, S. Nyberg, P. Algvere. 1990. Reversibility of cantaxanthin deposits within the retina. Acta Ophthalmol. (Copenh.), 68(5):607-611.

60. Mera Pharmaceuticals Inc. 1999. Technical report TR.3002.001. Astaxanthin as an antioxidant: a summary.

61. Mera Pharmaceuticals Inc. 1999. Technical report TR.3003.001. Astaxanthin and health: a summary.

Table 1: List of analyses in human safety study conducted on Mera Pharmaceuticals' *Haematococcus pluvialis* algal meal.[1]

Blood chemistry analyses

Serum glutamate pyruvate transaminase (SGPT)
Lactate dehydrogenase (LDH)
Glucose
Total protein
Total bilirubin
Blood urea nitrogen (BUN)
Creatinine
Total cholesterol
High-density lipoprotein (HDL) cholesterol
Triglycerides
Low-density lipoprotein (LDL) cholesterol (calculated)
Albumin
Globulin

Complete blood count (CBC)

White blood count (WBC)
Red blood count (RBC)
Hemoglobine (HGB)
Hematocrit (HCT)
Mean corpuscular volume (MCV)
Mean corpuscular hemoglobin (MCH)
Mean corpuscular hemoglobin concentration (MCHC)

Red cell distribution width (RDW)
Platelet count
Neutrophil (segs)
Lymphocytes
Monocytes
Eosinophils
Bsophils
Red blood cell morphology
Coagulation test (activated partial thromboplastin time, PTT)

Urinalysis tests

Color	pH
Appearance	Protein
Specific gravity	Glucose
Leukocyte esterase	Ketones
Nitrite	Urobilinogen
Blood	Bilirubin

Table 2. Levels of of astaxanthin in selected types of seafoods[8]

	Astaxanthin		
Seafood type	Content (mg/kg)	Free/esterified	Main isomer
Sockeye salmon	26-37	Free,esterified**	3*S*,3'*S*
Coho salmon	9-21	Free,esterified**	3*S*,3'*S*
Chum salmon	3-8	Free,esterified**	3*S*,3'*S*
Chinook salmon	8-9	Free,esterified**	3*S*,3'*S*
Pink salmon	4-6	Free,esterified**	3*S*,3'*S*
Atlantic salmon	3-11	Free,esterified**	3*S*,3'*S*
Rainbow trout	1-3	Free,esterified**	3*S*,3'*S*
salmon eggs	0-14	Esterified***	N.A.
Red seabream	2-14	Esterified***	N.A.
Red seabream eggs	3-8	N.A.	N.A.
Peneaus monodon	10-150	Esterified,free**	3*S*,3'*S*
Lobster		Esterified,free**	N.A.*
Krill	46-130	Esterified***	3*R*,3'*R*

Krill oil	727	Esterified***	3***R***,3'***R***
Crayfish meal	137	Esterified***	N.A.*
Artic shrimp	1160	Esterified***	3***S***,3'***S***
Haematococcus pluvialis	10,000-30,000	Esterified***	3***S***,3'***S***

* Most crustaceans studied appear to have mostly the 3***S***,3'***S*** form, unlike Krill.

** depending on tissues, free or esterified astaxanthin may be found

*** also contain a small proportion of free astaxanthin

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